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The effects of a soluble factor released by sensitized mononuclear cells incubated with *S. haematobium* ova on eosinophil migration

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Summary. Incubation of sensitized mononuclear cells from patients with schistosomiasis with specific antigen containing a suspension of viable *Schistosoma haematobium* ova resulted in the release of a soluble factor which was chemotactic for eosinophils. Production of this substance was dectectable at 24 h and demonstrated peak chemotactic activity after 2 days in culture. The chemotactic potential was dose-dependent and attracted eosinophils obtained from patients with schistosomiasis or allergic diathesis. Human neutrophil motility was unaffected by this chemoattractant. Preliminary studies demonstrated that the chemotactic factor is a heat-stable substance with peak activity associated with a molecular weight of approximately 42,000. These findings may reflect an *in vitro* correlate of cell-mediated immunity and may indicate a role played by the lymphocyte in the control of eosinophil function in human biology.

INTRODUCTION

Stimulated lymphocytes synthesize and release biologically active substances called lymphokines (Pick & Turk, 1972; Rocklin, 1976), which are regarded as soluble mediators of the immune response even though their *in vivo* significance has proved difficult to demonstrate (Valdimarsson & Gross, 1973). Certain lymphokines have been shown to be chemotactic for leucocytes (Altman, 1978). Lymphokines chemotactic for eosinophils *in vitro* have been described by several investigators. These include lymphocyte supernatants from antigen-stimulated guinea-pig lymph node cells (Cohen & Ward, 1971), a lymphocyte derived chemotactic factor from patients with Hodgkin's disease (Kay, McVie, Stuart, Krajewski & Turnbull, 1975) and an eosinophil stimulation promoter (ESP) produced by immune lymphocytes when stimulated with either specific antigen (PPD, or schistosomal egg antigen) or PHA (Colley, 1973; Greene & Colley, 1974, 1976). Kazura, Mahmoud, Karb & Warren (1975) adopted the ESP assay system for use with human cells. Their findings showed that incubation of purified human peripheral lymphocytes from patients with schistosomiasis with *Schistosoma mansoni* ova resulted in the secretion of a substance into the culture medium that stimulated migration of highly purified eosinophils from patients with or without schistosomiasis. Lymphocytes from normal donors cultured in this way with soluble egg antigens (SEA) did not produce ESP.

In view of these findings, a study was undertaken to verify the production of such a chemotactic substance

Abbreviations: ESP, eosinophil stimulation promoter; PHA, phytohaemagglutinin; SEA, soluble egg antigen; MN, mononuclear cells; MEM, minimal essential medium; EAS, endotoxin activated serum; PBS, phosphate-buffered saline.

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using ova of *Schistosoma haematobium* as antigen, and to characterise the nature of the increased locomotion. Mononuclear cells from patients with schistosomiasis were cultured with *S. haematobium* ova after which the supernatants were harvested and assayed for chemotactic activity. The present study investigated the kinetics of the production of this chemotactic factor, its specificity and effect on eosinophil random migration, chemokinesis and true directional motility. The factor was partially characterized by molecular sieve column chromatography.

MATERIALS AND METHODS

Leucoattractant production

Heparinized blood (5 units preservative free heparin, B.P. Evans, per ml of blood) was obtained from untreated patients with schistosomiasis and from normal donors. Thirty millilitres of whole blood was layered on 20 ml of Hypaque–Ficoll of specific gravity 1·078 and centrifuged at 400 *g* for 40 min (Böyum, 1968). The interphase containing mainly mononuclear cells (MN) was harvested and washed twice with Eagle's Minimal Essential Medium (MEM). Residual red blood cells (RBC) were lysed with ammonium chloride (Boyle, 1968) and the remaining MN cells washed twice and resuspended in MEM (buffered with HEPES, pH 7·4, containing 2 mM -glutamine, Flow Laboratories; and 300 units/ml each of penicillin and streptomycin, Glaxo Allenbury) to a final cell concentration of 10×10^6 MN/ml. Viability was greater than 95% as assessed by trypan blue exclusion.

Ova from untreated patients with *S. haematobium* infections were isolated from 24 h samples of urine by filtration through a 50 μm nylon mesh sieve under suction. The ova were then washed off the sieve and collected in a 1·7% sodium chloride solution followed by a further wash in this solution. The final wash contained equal volumes of MEM and 1·7% sodium chloride. Ova were resuspended to a final count of 300 ova/ml in MEM prior to incubation with MN cells. Viability was assessed prior to culture by observing miracidial flame cell movement. Only egg suspensions of > 90% viability were used for culture. Supernatants were obtained from cultures containing 3 ml of 10×10^6 MN/ml; 1 ml of 300 *S. haematobium* ova/ml and 1 ml of heat-inactivated (56° for 0·5 h) human AB serum.

Cultures were incubated at 37° in a humidified incubator containing 5% CO_2 and 95% air, after which the cultures were centrifuged and the supernatant stored at −20° until assayed for eosinophil chemotactic activity.

Eosinophil preparation

Heparinized whole blood was obtained from patients with eosinophilia of varying aetiology. Eosinophils were isolated as described previously by the method of Sher & Glover (1976). Cells were resuspended to a final concentration of 2×10^6 eosinophils/ml in MEM and were greater than 90% pure. Viability as demonstrated by trypan blue staining was 98%.

Cell motility studies

Studies of the effect of this lymphocyte-derived chemotactic factor on eosinophil chemotaxis were of two types. Firstly, the direct effect of this substance on eosinophil random migration and chemotaxis was measured by counting the number of eosinophils that had completely migrated to the lower surface of 8μ pore size filters (Millipore Corp., Bedford, MA) (transfilter assay) as described previously by Wadee, Anderson & Sher (1980). The second study employed the leading front method of Zigmond & Hirsch (1973) to measure the exact types of cell motility influenced. Filters were stained according to the method of Wadee & Sher (1978). The chemotaxis of eosinophils to the ESP-like supernatant was standardized to assess the specificity of the production of such supernatants and the optimum culture time and dose required for efficient cell migration.

Endotoxin activated serum

Fresh serum was activated with 500 μg/ml of bacterial lipopolysaccharide (*Escherichia coli* 0127: B8, Difco, Detroit, Mich.) (EAS). The mixture was incubated for 30 min at 37° and diluted four-fold with MEM for chemotaxis assays.

Neutrophil preparation

Heparinized whole blood was obtained from normal volunteers and 30 ml was layered onto 20 ml of Hypaque–Ficoll of specific gravity 1·078 and centrifuged at 400 *g* for 20 min (Böyum, 1968). The granulocyte and red blood cell pellet was resuspended to 37·5 ml in physiological saline and made up to 50 ml with 3% gelatin and the cell suspension mixed and sedimented at 37° for 20–40 min. The granulocyte-rich supernatant was removed and residual red cells lysed with ammonium chloride (Boyle, 1968) and the granulocytes (mainly neutrophils) washed once in a large

volume of MEM. Cells were resuspended to a final concentration of 3×10^6 neutrophils/ml.

Partial characterization of the chemotactic factor released by the interaction of sensitized lymphocytes and specific antigen

Two experiments were undertaken to partially characterize and investigate the nature of the chemotactic factor released by sensitized lymphocytes. These involved the effects of heat treatment, dialysis and separation by molecular sieve chromatography.

The effect of heat treatment and dialysis. An aliquot of the chemotactically active supernatant was heated in a 56° waterbath for 60 min, after which it was assayed for chemotactic activity using the previously determined optimal dose. Controls with untreated supernatants were run with each experiment. Two millilitre aliquots of supernatants were also dialysed against 200 ml of MEM at 4° for 24 h and assessed for eosinophilotactic activity thereafter.

Partial purification on Sephadex G-75. Sephadex G-75 (Pharmacia Fine Chemicals, Uppsala, Sweden) was packed in a K15/90 (Pharmacia) column. The column was equilibrated with phosphate-buffered saline (PBS) (pH 7·4) at a flow rate of 1 ml/min.

All fractionations were performed at 4°. For standardization, proteins of known molecular weights were used in which cytochrome C (from horse heart; mol. wt 12,384) lysosome (egg white mol. wt 14,300) pepsin (porcine stomach mucosa; mol. wt 34,700) (all from the Sigma Chemical Company) and bovine albumin (from Plasma; mol. wt 69,000; Armour Pharmaceutical Co.) served as reference standards. Molecular weight determinations were obtained by plotting the elution volume against the log (molecular weight) for the proteins (Andrews, 1964). Fraction collection was initiated when the protein solution was judged to have entered the column. Ten millilitres of the chemotactically active supernatant were concentrated to 1 ml in an Amicon U.M. 10 filter (Amicon Corp., Lexington, MA). This was then layered onto the column and the fractions collected in 1 ml volumes. Samples were pooled into 6 ml volumes and the active components further purified by a second passage through the same column. Absorbance at 280 nm was measured with an ultraviolet spectrophotometer (Unicam SP1800). The chemotactic activity of each fraction was determined by counting the number of eosinophils that had completed transfilter passage after 4 h of incubation.

RESULTS

Assessment of the specificity of the production of a lymphocyte derived factor

In order to determine if an eosinophil chemotactic factor was produced only by sensitized lymphocytes in the presence of specific antigen, a series of culture systems containing various combinations of lymphocytes and ova were incubated at 37° for 5 days after which the supernatants so obtained were harvested and assessed for chemotactic activity. Five types of culture conditions were examined. These consisted of: (a) patient's MN cells (schistosomiasis) + ova + heat-inactivated AB serum; (b) normal MN cells + ova + heat-inactivated AB serum; (c) patient's MN cells + heat-inactivated AB serum; (d) Normal Mn cells + heat-inactivated AB serum; (e) OVA + heat-inactivated AB serum.

The effect of the supernatants obtained from the above cultures on eosinophil migration was assessed by using transfilter assays. All supernatants were diluted to 25% prior to the assessment of chemotaxis. As can be seen in Fig. 1, only supernatants from systems containing sensitized MN cells and ova consistently increased eosinophil migration. Supernatants from all other combinations of cells in culture failed to induce significant eosinophil migration.

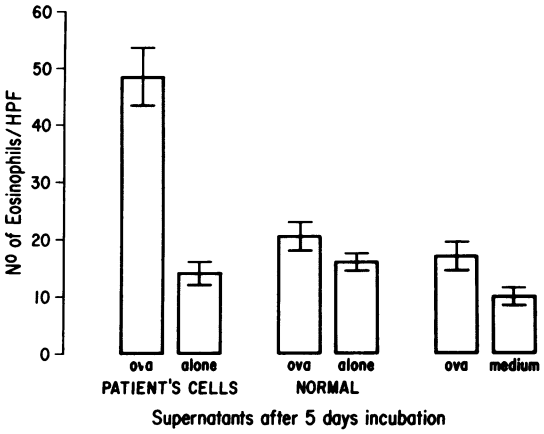

Figure 1. The effects of supernatants obtained from combinations of *Schistosoma haematobium* ova and MN cells from patients with Schistosomiasis on eosinophil chemotaxis. Migration was compared to the effects of supernatants from control cultures obtained by incubating mononuclear cells in the following ways: schistosomiasis patient's cells alone, normal cells + ova; normal cells alone, ova or medium alone. (Mean ± SEM of ten individual experiments.)

Assessment of chemotactic factor requirement where a lymphocyte-derived chemotactic factor provides the leucotactic stimulus

The effect of varying concentrations of supernatants from cultures containing lymphocytes from patients with schistosomiasis and *S. haematobium* ova on eosinophil motility was investigated in these studies. Aliquots (0·9 ml) of this chemoattractant varying from undilute to a 1/32 dilution (undilute, ½, ¼, 1/8, 1/16, 1/32) were placed in the lower chamber and the number of eosinophils completing transfilter passage enumerated after 4 h of chamber incubation. A maximum eosinophil chemotactic response was obtained at a leucoattractant concentration of 25% (Fig. 2).

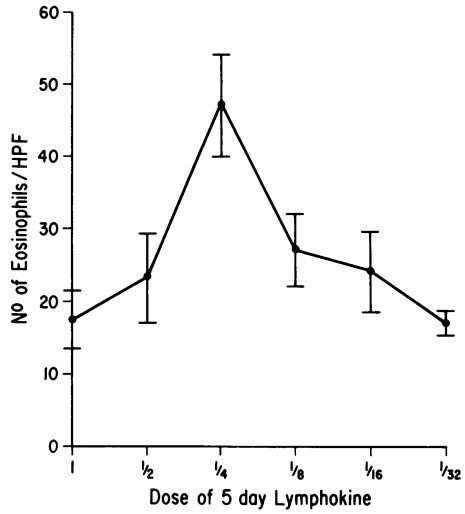

Figure 2. Dose kinetics of chemotactic supernatants. Supernatants from 5 day cultures were serially diluted and assessed for eosinophil chemotactic activity. Cultures were diluted in T-HBSS. (Mean ± SEM of ten individual experiments.)

Assessment of duration of culture incubation for attaining optimal eosinophil chemotaxis

In order to examine the effects of the length of time of culture (of sensitized lymphocytes with specific antigen) on eosinophil motility, cultures were incubated at 37° from 24 h to 5 days (1, 2, 3, 4 and 5 day cultures). After centrifuging the cellular elements, supernatants from each culture were harvested and stored at −20° until assessed for chemotactic activity. The effects of culture incubation time on the chemotactic potency of the respective supernatants is shown in Fig. 3. Two day culture supernatants appeared to induce a maximal eosinophil chemotactic response (66 ± 9 cells/HPF). Migration towards supernatants of 3, 4 and 5 day cultures, however, appeared to be reduced possibly due to partial degeneration with time.

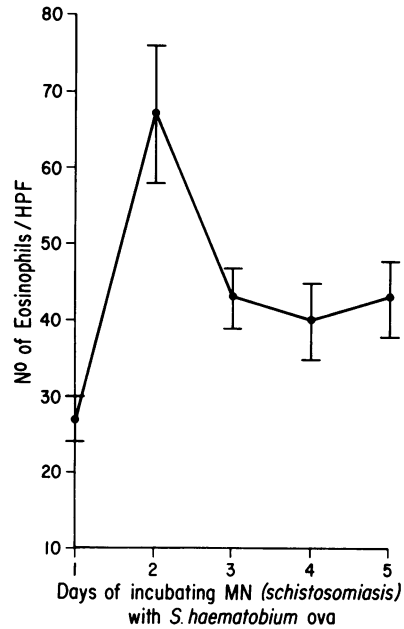

Figure 3. Kinetics of chemotactic factor production. Sensitized MN cells were incubated with *Schistosoma haematobium* ova for varying periods of time after which the supernatants were harvested and assessed for chemotactic activity. (Mean ± SEM of ten individual experiments.)

Assessment of eosinophil random migration chemokinesis and true chemotaxis in the presence of 2 day culture supernatants derived by incubating sensitized lymphocytes with specific antigen

The effect of 2 day culture supernatants on eosinophil motility (random migration, chemokinesis and chemotaxis) was assessed using the checkerboard technique of Zigmond & Hirsch (1973). Varying concentrations of the chemoattractant (1·5%, 3%, 6%, 12%) were placed above and below the filter to assess true directional motility. Eosinophil motility was assessed by measuring the distance traversed (μm) by the leading front line of cells. Table 1 shows that the leucoattractant is chemokinetic (stimulated random movement in the absence of a gradient, within the diagonal). In addition, directional motility also appeared to be

Table 1. The effects of varying concentrations of supernatants obtained from culturing sensitized lymphocytes with specific antigen on eosinophil chemokinesis and true chemotaxis

% Supernatant above filter	%Supernatant below filter				
	0	1·5	3	6	12
0	**23**				
1·5		**25**	35 (26)	39 (28)	44 (34)
3			**30**		
6				**37**	
12					**40**

Figures (in bold type) along the diagonal from upper left to the lower right show the distance migrated (μm) in increasing concentrations of the leucoattractant in the absence of a concentration gradient. The figures in parentheses are estimates of what migration would have been in each of the tests, assuming that the cells detected the absolute concentration of the chemoattractant and not the gradient. Above the diagonal, cells are moving in a positive gradient. The true chemotactic values (TCV) in positive gradients: 15/3, 15/6, 15/12 (concentration above/concentration below the filter) are 9, 11 and 10, respectively. These were calculated by subtracting the expected values (in parentheses) from the observed values next to them.

increased in the presence of all positive gradients assayed (true chemotactic values, TCV).

Partial characterization of the chemotactic factor (The effects of heat treatment, dialysis and molecular weight estimation)

Table 2 shows the eosinophil migratory response to the heat-treated chemoattractant. Chemotaxis was unaffected by heating the supernatant at 56° for 1 h. Dialysis against culture medium showed no significant

Table 2. The effect of heat treatment and dialysis of chemotactic supernatants on eosinophil chemotaxis (mean ± SEM of three experiments)

Nature of treatment of supernatant	No. of eosinophils/HPF
Heat treatment (supernatants incubated at 56° for 1 h)	58 ± 7
Dialysis against MEM at 4° for 24 h	56 ± 8
Controls (untreated supernatants)	64 ± 6

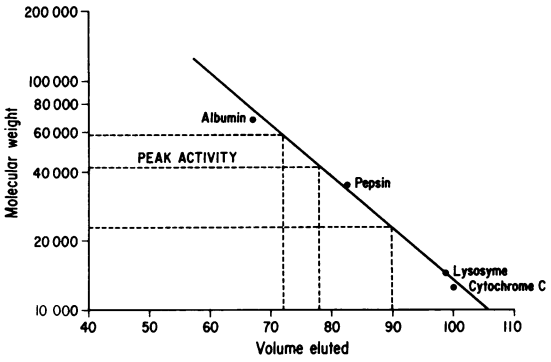

Figure 4. The relationship between proteins of known molecular weight and elution volumes on a Sephadex G-75 column equilibrated with PBS (pH 7·4).

loss of chemotactic activity. Figure 4 (a mean of five experiments) shows plots of elution volume from a Sephadex G-75 column against log (molecular weight) for cytochrome C, lysosyme, pepsin and bovine albumin.

The experimental plots lie close to a straight line. The molecular weight estimation of the active fractions were read off this standard curve by extrapolating the chemotactic fractions eluted against the log molecular weight. The chemotactically active component appears to lie in the molecular weight range of 23,000 and 58,000 with peak activity at 42,000. Absorbance at 280 nm indicates that the activity lies in the smaller protein fraction (Fig. 5).

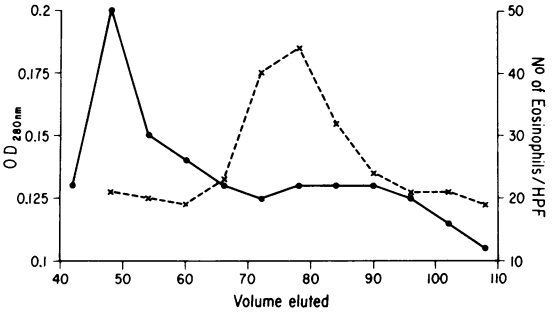

Figure 5. Sephadex G-75 column chromatography of supernatant fluids from 2 day cultures of sensitized lymphocytes and specific antigen. The protein content of the various fractions were estimated by the absorbance on 280 nm, on the left (•). Eosinophil chemotactic activity of undiluted eluted fractions is indicated on the right (×). (Mean of five experiments.)

The effect of supernatants from cultures containing sensitized lymphocytes and specific antigen on neutrophil migration

In order to ascertain whether these supernatants were specifically chemotactic for eosinophils, studies were undertaken using human neutrophils as target cells. Neutrophil motility was studied using 5 μm pore size filters and chambers were incubated at 37° for 3 h. These differences in cell number and chamber incubation times were unavoidable as previous studies from this laboratory (Anderson, personal communication) have shown maximal neutrophil chemotactic response to endotoxin activated serum (EAS) under these conditions. A 25% concentration of EAS was run consecutively with each assay of supernatant activity. Random motility was examined by placing 0·1% AB serum on both sides of the filter. The normal cell counts in these systems were 172 ± 19 (mean $\pm$ SEM) neutrophils/HPF for chemotaxis and 26 ± 8 (mean $\pm$ SEM) cells/HPF in random motility studies. A range of the supernatant (undilute to 1/64) was assessed for chemotactic potency. No appreciable neutrophil movement towards the sensitized lymphocyte derived chemoattractant was observed (Fig. 6). Furthermore, regardless of the concentration used, chemotaxis did not rise significantly above that obtained in random motility studies.

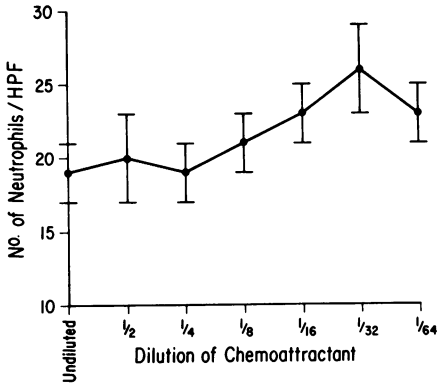

Figure 6. The effect of 2 day culture supernatants on neutrophil chemotaxis. Supernatants were diluted serially in T-HBSS. (Mean $\pm$ SEM of five experiments.)

DISCUSSION

The observations reported herein indicate that eosinophil migration can be stimulated by a product resulting from the interaction of sensitised lymphoid cells and specific antigen. These findings are very similar to those reported by Colley (1973) who showed an eosinophil chemotactic substance (eosinophil stimulation promoter, ESP) produced by lymph node cell cultures stimulated with antigens to which the donor mice had been specifically sensitized. The findings presented here also augment those of Kazura *et al.* (1975) who showed that peripheral lymphocytes from patients with schistosomiasis when incubated with egg antigen, secreted the eosinophil stimulation promoter which enhanced the migration of purified eosinophils from patients with or without schistosomiasis. Exposure of eosinophil-rich peritoneal cell populations from *S. mansoni* infected mice to a soluble schistosomal egg antigen (SEA) has also been reported to stimulate eosinophil migration (Colley, 1973). In the present study, however, supernatants from cultures containing *S. haematobium* ova alone were unable to induce appreciable eosinophil migration. These findings are not totally in disagreement with those reported by Colley (1973) as the concentration of egg antigen in these culture supernatants may have been too low to induce appreciable migration. Similar findings have been reported by Kazura *et al.* (1975) who showed that eosinophil migration was not enhanced when SEA was added in an amount equivalent to that of the antigen in cultures (SEA + sensitized lymphocytes).

Dose response curves have shown a maximal eosinophil chemotactic response to a 25% concentration of this substance. It is difficult to compare these findings to those reported by Greene & Colley (1974) who showed a decline in migration with an increase in the dilution factor, as the methods used for assaying eosinophil migration in these two systems are different. Nevertheless, certain properties of this chemoattractant appear to be similar to those reported by previous authors. Colley (1973) showed that the overwhelming majority of cells stimulated to migrate by ESP out of eosinophil rich peritoneal exudates (EPE) were eosinophils. In these systems, however, the cell suspensions consisted of approximately 65% eosinophils, 20% macrophages, 10% lymphocytes and 5% neutrophils. Because of the relatively low numbers of neutrophils used, it is not possible from this study alone to consider that ESP is a specific eosinophil chemoattractant. The use of highly purified populations of eosinophils and neutrophils (>90%) in the present study, however, shows quite clearly that supernatants derived from lymphocyte cultures enhance the

migration of eosinophils only, and have no appreciable effect on neutrophil chemotaxis. Furthermore, this substance has also been shown to stimulate eosinophil chemokinesis and to induce a true directional response of these cells as assessed by the Zigmond & Hirsch assay (1973). Similar findings have been reported by Colley (1973) and Lewis & Colley (1977). Finally, molecular weight estimations by column chromatography has also shown this substance to be very similar to the ESP reported by Colley (1973). As the properties of this substance has been demonstrated to be similar to the ESP described by Colley and co-workers, it is tentatively classified as being an eosinophil stimulation promoter obtained from sensitized human lymphoid cells.

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